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cis-12-oxo-phytodienoic acid represses *Arabidopsis thaliana* seed germination in shade light conditions

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Highlight

OPDA acts in addition to ABA to repress seed germination under far-red light conditions. The response to both these phytohormones is integrated by MFT, a negative regulator of germination.

Abstract

Light-dependent seed germination is induced by gibberellins (GA) and inhibited by abscisic acid (ABA). The widely accepted view of GA/ABA ratio controlling germination does not however explain the fact that seeds deficient in ABA still germinate poorly under shade light conditions that repress germination. In Arabidopsis, MOTHER-OF-FT-AND-TFL1 (MFT) acts as a key negative regulator of germination, modulating GA and ABA responses under shade light conditions. Under full light the oxylipin cis-12-oxo-phytodienoic acid (OPDA), a precursor of the stress related phytohormone jasmonic acid, interacts with ABA and MFT to repress germination. Here, we show that, under shade conditions both OPDA and ABA repress germination to varying extents. We demonstrate that the level of shade induced *MFT* expression influences the ability of OPDA and/or ABA to fully repress germination. We also find that *MFT* expression decreases with seed age and this again correlates with the response of seeds to OPDA and ABA. We conclude that OPDA plays an essential role alongside ABA in repressing germination in response to shade light and the combined effect of these phytohormones is integrated to a significant extent through *MFT*.

Key words

OPDA, ABA, MFT, phytochrome, FR-light, shade, seed germination

Abbreviations

ABA:	abscisic acid
FR:	Far Red
GA:	giberellins
hai:	hours after imbibition
JA:	jasmonic acid
JA-Ile:	jasmonic acid -isoleucine
OPDA:	*cis*-12-oxo-phytodienoic acid
R:	Red

Introduction

Timing of seed germination is one of the most important decision points in the life cycle of a higher plant. The environmental conditions under which a seed germinates are critical for survival and consequently control mechanisms that integrate environmental cues such as temperature and light quality have evolved to control the timing of germination in a number of species (Smith, 2000; Linkies *et al.*, 2010; Kendall *et al.*, 2011; Lee and Lopez-Molina, 2012). These cues regulate accumulation and perception of gibberellins (GA) and abscisic acid (ABA) phytohormones, which promote and repress seed germination respectively (Seo *et al.*, 2006; Kendall *et al.*, 2011; Shu *et al.*, 2016). ABA, acting through ABA response transcription factors such as ABA-INSENSITIVE3 (ABI3), ABI4 and ABI5 (Finkelstein *et al.*, 1998, 2000; Clerkx *et al.*, 2003), accumulates during seed development to induce a physiologically dormant state, whereby newly formed seeds do not germinate even under favourable environmental conditions (Graeber *et al.*, 2012; Chahtane *et al.*, 2017). Seeds gradually lose their dormancy through an after-ripening process after which they can germinate if environmental conditions are favourable (Holdsworth *et al.*, 2008; Smith, 2000; Jiao *et al.*, 2007).

In many plant species, including *Arabidopsis thaliana*, the probability of seedling establishment is generally greater if germination occurs under direct sun (white) light, which is rich in the red (R) wave length; rather than under-the-canopy light (shade), which is rich in far-red (FR) (Lee and Lopez-Molina, 2012). In photoblastic seeds, phytochrome photoreceptors distinguish between these different light conditions on the basis of their R and FR light intensities and ratios (Shinomura, 1997; Smith, 2000; Quail, 2002; Jung *et al*., 2016). Excess R triggers GA accumulation and germination, whereas excess FR, typical of shade light, triggers ABA accumulation and a block in germination (Oh *et al.*, 2006; Seo *et al.*, 2006; Piskurewicz *et al.*, 2008). Arabidopsis has five phytochromes, (phyA-E); Clack *et al.*, 1994), with phyB being the main promoter of germination under sun light; while phyA is responsible for germination under-the-canopy light (Shinomura *et al.*, 1994). Both phyA and phyB are synthesized as inactive proteins and become active in a light-quality dependent manner. However, while short pulses of R and FR light are sufficient to activate and deactivate phyB respectively; longer exposures to R and FR activate phyA (Reed *et al.*, 1994; Shinomura *et al.*, 1994). Furthermore, compared to phyB, phyA accumulates at high levels only after relatively long periods of seed imbibition (Lee *et al.*, 2012).

Upon activation, both phyA and phyB induce degradation of the transcription factor PHYTOCHROME INTERACTING FACTOR1 (PIF1, formerly known as PIL5) (Shen *et al.*, 2005; Park *et al.*, 2012). Upon phytochrome inactivation, PIF1 accumulates and regulates transcription of many genes, including SOMNUS (SOM), which encodes a CCCH- type zinc finger protein that is part of the phytochrome signal transduction pathway controlling genes involved in regulating ABA and GA levels ultimately leading to high ABA:GA ratios to repress germination (Oh *et al.*, 2004; Oh *et al.*, 2007; Kim *et al.*, 2008; Kim *et al.*, 2016; Park *et al.*, 2011). We showed recently that PIF1 and SOM also promote *MOTHER-OF-FT-AND-TFL1* (*MFT*) expression and that MFT plays a key role in repressing germination by modulating ABA and GA responses (Vaistij *et al.*, 2018). Furthermore, PIF1 stimulates the expression of *GAI* and *RGA*, which encode growth repressing DELLA proteins (Oh *et al.*, 2004; Oh *et al.*, 2007; Piskurewicz *et al.*, 2008; Piskurewicz *et al.*, 2009). GA promotes germination by targeting destruction of the DELLA proteins through the 26S-proteasome. Under FR conditions, the DELLA proteins RGL2, GAI, and RGA repress germination by stimulating the expression of ABA biosynthetic genes, further increasing the ABA:GA ratio (Piskurewicz *et al.*, 2008; Piskurewicz *et al.*, 2009; Lee *et al.*, 2012).

The phytohormone jasmonic acid (JA) and its precursor *cis*-12-oxo-phytodienoic acid (OPDA) are oxilipins derived from linolenic acid (Wasternack and Song, 2016). The biologically active conjugated JA-isoleucine (JA-Ile) form is involved in responses to biotic and abiotic stress as well as in many other biological processes including seed germination (Linkies and Leubner-Metzger, 2012; Wastermack and Hause, 2013; Wastermack and Strnad, 2015; Singh *et al.*, 2017). OPDA also exhibits signalling properties, some of which are shared with JA-Ile, but others are distinct (Goetz *et al.*, 2012; Guo *et al.*, 2014; Savchenko and Dehesh, 2014; Bosh *et al.*, 2014; Dave *et al.*, 2011). Previously we characterised the role of oxilipins in seed dormancy. We did this by analyzing mutant seeds defective in: (i) *ALLENE OXIDE SYNTHASE* (*AOS*), which encodes a cytochrome P450 oxidase enzyme involved in one of the final steps of OPDA biosynthesis inside plastids (Park *et al.*, 2002); (ii) *PXA1* (also known as *CTS* and *PED3*), which encodes an ABC-type transporter that imports OPDA into peroxisomes (Zolman *et al.*, 2001; Footitt *et al.*, 2002; Hayashi *et al.*, 2002); and (iii) *12-OXOPHYTODIENOIC ACID REDUCTASE* (*OPR3*), which is involved in the conversion of OPDA to JA in peroxisomes (Stintzi and Browse, 2000). It has been determined that seeds of the *aos* mutant, which is compromised in OPDA and JA/JA-Ile

accumulation, are less dormant than wild-type seeds; whereas seeds of the *pxa1-1* and *opr3-1* single mutants, which over-accumulate OPDA but are deficient in JA/JA-Ile, are more dormant (Chehab *et al.*, 2011; Dave *et al.*, 2011; Dave *et al.*, 2016). These observations led us to conclude that OPDA specifically acts as a dormancy-promoting factor. In the present work we investigated the role of OPDA in the FR triggered repression of germination of after-ripened seeds. We show that endogenous OPDA in seeds plays a key role alongside ABA to repress germination under shade conditions through an MFT modulated process.

Material and methods

Growth conditions and biological materials. Plants were grown in a greenhouse supplemented with artificial light to give a photoperiod of 16 h light at a temperature of 20-22 °C. Seeds were harvested when plants stopped flowering and siliques started to dehisce. In all experiments, except for that involving extended after-ripening shown in Figure 6, seeds were after-ripened for no longer than 8 weeks. All mutant lines used in this study were described previously: *aos* (Park *et al.*, 2002); *opr3-1* (Stintzi and Browse, 2000); *opr3-3* (Chini *et al.*, 2018); *aba2-1* (Leon-Kloosterziel *et al.*, 1996); *mft-2* (Xi *et al.*, 2010); *rgl1-1 rgl2-2 gai-6 rga-2* (della4) (Cao *et al.*, 2005); *cyp20-3* (Park *et al.*, 2013).

Germination assays. Seeds were vapour-phase sterilized by exposure to chlorine gas in a sealed glass container to at least three hours, the gas having been produced by mixing 100 ml of bleach with 3 ml of concentrated HCl. Sterilized seeds were plated on water agar (0.9 % w/v) and allowed to imbibe under low light for 4 hours and then LED- irradiated with FR (4.5 μmol $m^{-2}.s^{-1}$) and R (20 μmol.$m^{-2}.s^{-1}$) as indicated in Figure 1A. After FR/R, FR and FR48 treatments plates were wrapped in foil and kept at 20 °C. Germination was scored on the basis of radical emergence of 50-100 seeds per replica. In experiments where germination assays were conducted with ABA (Sigma-Aldrich), OPDA (Larodan), paclobutrazol (Sigma-Aldrich) or norflurazon (Sigma-Aldrich) the appropriate amounts of these compounds were included in the water agar media.

Phytohormone analyses. At least four biological replicates of 100 mg of seeds were imbibed and light-treated as depicted in Fig. 1A, all imbibed seeds were collected and phytohormones extracted as described previously (Dave *et al.*, 2011). Extracts were resuspended in methanol and 2 μL injected and analyzed on an ultraperformance liquid chromatography (UPLC)-MS system consisting of an Acquity

UPLC I-Class system (Waters) coupled to a TSQ Endura triple quadrupole mass spectrometer (Thermo Scientific). Chromatographic separation of phytohormones was performed at 40°C on a Waters Acquity C18 BEH column (50 mm x 2.1 mm x 1.7µm particle size), using a binary gradient of mobile phases with A = water + 0.1% (v/v) acetic acid and B = acetonitrile + 0.1% (v/v) acetic acid. The gradient elution program was as follows: 0 - 0.61 min isocratic 10% B; 0.61 - 2.34 min to 100% B; 2.34 - 2.82 min isocratic 100% B; 2.82 - 2.83 min to 10% B; 2.83 - 3.30 min isocratic 10% B. Eluted compounds were ionized on the mass spectrometer using a heated electrospray (HESI) source from 0.2 - 2.5 min, in negative ion mode (spray 2500 V; sheath N2 gas 60 units, Aux N2 gas 20 units, sweep N2 gas 2 units, vaporizer 400 °C, ion transfer tube 380°C). Precursor and product ions were filtered through Q1 and Q3 respectively at a mass resolution of 1.2 Da and at a fixed dwell time of 35 ms per transition. The following precursor - product ion transitions were programmed using Thermo Xcalibur software in SRM mode: ABA 263.2 - 153.2; d6-ABA 269.2 - 159.2; JA 209.2 - 59.4; GA4 331.2 - 287.1; JA-Ileu 322.2 - 130.2; prostaglandin A1 317.2 - 273.2; OPDA 291.2 - 165.2. For ABA and GA4, product ion peak area ratios relative to their respective deuterated analogs added as internal standards were used to construct calibration curves and calculate concentrations. For all other compounds, prostaglandin A1 was used as the reference internal standard. All standards were obtained as described in Dave et al (2011).

Gene expression analysis. RNA extractions were performed as described previously (Vaistij *et al.*, 2013). Standard protocols were used for RQ1 RNase-Free DNase treatments (Promega), cDNA synthesis (SuperScript®II, Invitrogen) and qPCRs (iTaq^{TM}Universal Syber® Green, Bio-Rad). Transcript abundance of a stable endogenous control (*UBQ11*; see Sup. Fig. 1) was used for normalization and gene expression was expressed as a fold change relative to the control sample. Primer sequences for the qPCRs are described in Table S1.

Results and discussion

Differential expression of *AOS* and *OPR3* does not result in OPDA accumulation in FR-treated seeds. In a recent RNAseq-based transcriptomic analysis, we observed that *AOS* expression is FR induced (Vaistij *et al.*, 2018; Sup. Fig. 1). This suggested that, as for ABA, OPDA biosynthesis is induced by FR light. In order to validate the transcriptomic data, we performed RT-qPCR to quantify transcript abundance of *AOS* and *OPR3* in wild-type (Col) after-ripened seeds treated with two consecutive short pulses of FR and R light (FR/R) or a single FR pulse (Fig. 1A) in order to activate and

178 deactivate phyB, respectively. Consistent with the RNAseq data, we found that *AOS* expression was
179 approximately 4 to 5-fold higher in FR-treated seeds compared to FR/R controls at 12 and 24 hours-
180 after-imbibition (hai) (Fig. 1B). In contrast, the *OPR3* transcript abundance was unaffected at 12 hai and
181 was less than 2-fold lower in FR-treated seeds compared to the FR/R treatment at 24 hai (Fig. 1C).
182 These results at the level of gene expression prompted us to assess OPDA levels in FR/R and FR-treated
183 wild-type seeds. We also measured ABA levels and analysed FR-treated *mft-2* seeds. As previously
184 reported, ABA levels were increased in FR-treated wild-type seeds compared to FR/R-treated seeds, and
185 in *mft-2* seeds compared to wild-type seeds under FR light conditions (Seo *et al*., 2006; Vaistij *et* al.
186 2018; Fig. 1D). Surprisingly, despite the differential *AOS* expression, we detected no significant changes
187 in OPDA accumulation in wild-type and *mft-2* seeds under FR light conditions compared to the
188 respective controls (Fig. 1E). We also measured JA and JA-Ile levels in wild-type seeds upon FR/R and
189 FR treatments (24 hai) but found no significant changes in their accumulation (Sup. Fig. 2). Thus, our
190 findings indicate that total OPDA, JA and JA-Ile accumulation in seeds is not regulated by light quality.
191 However, we cannot rule out the possibility that light may affect oxylipin accumulation in a localised
192 cell specific manner, which would not be detected by the whole seed phytohormone extraction
193 methodology we have available. Future work could explore detection methods that allow more localised
194 mapping of phytohormones within seed tissues.
195
196 **OPDA acts in addition to ABA to repress seed germination in the shade**. Although we could not
197 detect changes in OPDA levels in FR-treated seeds we were curious as to whether oxylipins played a
198 role in regulating germination under FR light conditions. Hence, we analysed germination of after-
199 ripened *aos* mutant seeds, which are deficient in OPDA, JA and JA-Ile (Dave *et al*., 2011). We observed
200 that, as expected, wild-type and *aos* seeds germinate at high rates upon FR/R treatment and at very low
201 rates (routinely less than 10 %) under FR conditions (Fig. 2A). Presumably, the well documented ABA
202 inhibition of germination in response to FR light causes inhibition of germination in these oxylipin
203 mutants as well as in wild-type seeds. We then used the ABA biosynthesis inhibitor norflurazon to
204 further investigate a possible interaction between OPDA and ABA. We found that blocking ABA
205 biosynthesis in the wild-type background does not rescue the FR block on wild-type seed germination
206 (Fig. 2A), which is consistent with a previous report by Lee *et al*. (2012). This implies that something
207 else is blocking germination in response to FR treatment when ABA biosynthesis is impaired.
208 Interestingly, we found that germination of FR-treated *aos* seeds in the presence of norflurazon

germinated at high rates, in a dose dependent manner, suggesting a role for oxylipins in regulating the FR response, at least when ABA biosynthesis is compromised (Fig. 2A). We also assessed germination of two *OPR3* mutant alleles, *opr3-1* and *opr3-3*. Seeds of both *opr3-1* and *opr3-3* accumulate OPDA and ABA at similar levels as wild-type but are both significantly impaired in JA and JA-Ile accumulation (Sup. Fig. 2). The *opr3-1* and *opr3-3* seeds do accumulate very low amounts of JA-Ile, possibly due to the activity of the recently reported OPR3-independent biosynthetic pathway (Chini *et al., 2018*; Wasternack and Hause; 2018). We found that, similar to wild-type, both *opr3-1* and *opr3-3* seeds germinated at high levels under FR/R conditions; and at extremely low levels upon FR-light treatments even in the presence of norflurazon (Fig. 2B). This indicates that OPDA rather than JA/JA-Ile acts to repress germination under FR light, as was found to be the case under white light conditions (Dave *et al.*, 2011; Dave *et al.*, 2016).

In order to further validate our observations, we also analysed *aos* seeds in the ABA biosynthesis deficient *aba2-1* mutant background (Leon-Kloosterziel *et al.*, 1994). We found that while *aos* and *aba2-1* single mutant seeds do not germinate, *aos aba2-1* double mutant seeds germinate at high rates under the normally repressing FR light conditions (Fig. 2C). These results support the view that blocking the biosynthesis of both OPDA and ABA is required to allow germination of after-ripened seeds under strong phyB-deactivating FR light conditions. Consistent with this, we determined that while application of either OPDA or ABA represses germination of FR-treated *aos aba2-1* seeds (Fig. 2D), exogenous JA has no significant effect on germination of the double mutant (Sup. Fig. 3). Overall, these results demonstrate that the strong repression of germination imposed by the FR light treatment is alleviated when the accumulation of both OPDA and ABA is compromised.

PhyA-dependent germination of *aos aba2*-1 double mutant and control seeds was also assessed after 48 h of continuous FR light treatment followed by four days in the dark (FR48; Fig. 1A). Under these conditions phyA and phyB are activated and deactivated respectively. As expected, we observed that FR48-treated wild-type seeds germinated at higher rates than seeds given just a short FR pulse; *aos* and *aba2-1* single mutants germinate at higher levels than the wild-type control seeds; and *aos aba2-1* double mutant seeds germinate at even higher rates than the single mutants (Fig. 2C). There are similarities between the role of ABA in this study and the one described by Lee *et al.* (2012), which showed that attenuation of ABA-dependent responses is required to promote phyA-dependent

240 germination. Overall, these results demonstrate that disruption of either ABA or OPDA biosynthesis
241 results in increased phyA-dependent seed germination.
242
243 To gain insight into the interplay between OPDA, ABA and GA in the control of germination in
244 response to FR light, we analyzed the GA requirement of the *aos aba2-1* double mutant under FR light
245 conditions by treating seeds with the GA-biosynthesis inhibitor paclobutrazol. We observed that under
246 these conditions *aos aba2-1* seeds failed to germinate (Fig. 3A). It is well established that FR conditions
247 are associated with low GA/ABA ratios in seeds (Seo *et al.*, 2006), and that DELLA factors play a
248 critical role in stimulation of ABA biosynthesis under these conditions (Piskurewicz *et al.*, 2009). Thus,
249 we also analysed *rgl1-1 rgl2-2 gai-6 rga-2* quadruple mutant seeds (*della4*) and determined that, as is
250 the case with exogenous ABA, exogenous OPDA represses the high levels of germination that these
251 seeds exhibit under FR light (Fig. 3B). These results suggest that OPDA acts downstream of DELLA
252 factors to repress germination under FR light conditions, which is similar to what has been previously
253 reported for ABA (Piskurewicz *et al.*, 2009). However, it is also possible that OPDA and DELLAs have
254 parallel pathways that repress germination under FR conditions.
255
256 **OPDA and/or ABA repression of germination correlates with *MFT* expression**. Previously, we
257 established that OPDA has no effect on repressing germination of *mft-2* mutant seeds under white light
258 (Dave *et al*., 2016); here we show that OPDA also fails to repress *mft-2* germination under FR light
259 conditions (Fig. 3C). This strengthens our view that OPDA acts upstream of MFT. In addition, we
260 previously demonstrated that *MFT* expression is induced in a PIF1- and SOM-dependent manner under
261 FR conditions (Vaistij *et al.*, 2018). Here we show that the transcript levels of *PIF1*, *SOM* and *MFT* are
262 strongly increased upon FR-treatment compared to FR/R in wild-type seeds, and this increase is
263 intermediate under FR48 conditions (Fig. 4A). Comparing germination rates of wild-type (Fig. 2C) with
264 *MFT* expression under FR/R, FR and FR48 (Fig. 4A) reveals a negative correlation. These observations
265 led us to hypothesise that a deficiency in both ABA and OPDA is required to overcome the strong
266 germination inhibitory effects of FR conditions when *MFT* is highly expressed; whereas under phyA-
267 dependent germination conditions (FR48), where *MFT* expression is reduced, the absence of either ABA
268 or OPDA is sufficient to alleviate the block on germination. To test this hypothesis we assessed wild-
269 type, *aos*, *aba2-1* and *aos aba2-1* seeds under FR48 light conditions and determined that *MFT*
270 expression is reduced in the mutant backgrounds, with the strongest effect seen in *aba2-1* and *aos aba2-*

1 (Fig. 4B). Taken together, these observations lead us to conclude that MFT integrates both ABA and OPDA signalling pathways in order to repress germination in the shade: The necessity for just one or both of ABA and OPDA for repression of germination depends on endogenous levels of MFT. It is worth noting however, that although *MFT* expression is at a similar low level in *aba2-1* and *aos aba2-1* (Fig. 4B), the germination rate of the double mutant seeds is higher than the single mutant seeds (Fig. 2C) suggesting that factors other than MFT also play a role.

OPDA and/or ABA repression of germination correlates with seed age. Lee *et al.* (2012) observed that blocking ABA biosynthesis by norflurazon treatment of wild-type seeds does not alleviate the repression of germination by FR light, which is in agreement with our observations (Fig. 2A). However, Seo *et al.* (2006) reported that ABA biosynthesis deficient mutant seeds germinate partially under FR conditions, which contrasts with our analyses of *aba2-1* seeds (Fig. 2B). While the report of Seo *et al.* (2006) did not indicate the age of after-ripened seeds used in their germination assays, our study and that of Lee *et al.* (2012) were performed with seeds not older than 8 weeks from the time of maturation/collection. This led us to question whether seed age may influence the sensitivity to OPDA and ABA in terms of germination repression under shade light conditions. In order to address this, seeds after-ripened for more than nine months were treated with FR/R and FR light. Interestingly, germination rates of long-term after-ripened norflurazon-treated wild-type seeds and *aos* seeds (not treated with norflurazon) were 75 % and 40 %, respectively, under FR conditions (Fig. 5A and 5B). These germination rates are much higher than those found routinely for the same treatments of short-term (less than 8 weeks) after-ripened seeds (Fig. 2A). Noteworthy also is the fact that long-term after-ripened wild type seeds are still very responsive to the germination repressing effects of FR light. Taken together these results demonstrate that as seeds age there is a necessity for both ABA and OPDA to block germination under FR light whereas in younger after-ripened seeds either one is sufficient. A possible explanation for this might be that aged seeds are less sensitive to dormancy-promoting factors than younger seeds (Holdsworth *et al.*, 2008; Holman *et al.*, 2009). We have previously shown that MFT is a strong promoter of seed dormancy (Vaistij *et al*., 2013). Therefore, we hypothesized that MFT may be involved in the age-dependent requirement of OPDA and/or ABA to repress germination. To test this we assessed *MFT* expression in young (less than 8 weeks) and old (more than 9 months) wild-type seeds treated with FR light and found that *MFT* expression is reduced in the older seeds (Fig. 5C). This parallels the negative correlation between *MFT* expression levels and OPDA and/or ABA requirements

of young seeds under FR and FR48 conditions (Fig. 2B and 4A). These observations further support our conclusion that MFT integrates both ABA and OPDA signalling pathways in order to repress germination and that both environmental conditions such as light quality or developmental factors such as seed age playing an important role in regulating germination through MFT expression. As seeds age other changes may also occur, such as decrease in phytohormone levels. While we have demonstrated an important role for MFT, we cannot rule out the possibility of other factors also having an effect on the sensitivity to OPDA and ABA under FR light conditions.

CYP20-3 is involved in OPDA signalling in seeds. The crosstalk between ABA and OPDA may influence their abundance as well as their associated signalling pathways. We established previously that both gene expression and protein accumulation of the ABI5 transcription factor are induced by OPDA (Dave *et al.*, 2011; Dave *et al.*, 2016). It has also been shown that the forever-dormant phenotype of the OPDA over-accumulating *ped3*-3 mutant is dependent on ABI5 (Kanai *et al.*, 2010), and that ABI5 accumulation is induced by FR light (Piskurewicz *et al.*, 2009). However, despite this apparent involvement of ABI5 in signalling both ABA and OPDA, *abi5* mutant seeds fail to germinate under FR light (Lee *et al.*, 2012). This indicates that factors other than ABI5 are involved in signalling the ABA- and OPDA-triggered repression of germination under shade light conditions. Interestingly, it has been shown that, in wounded leaves, CYCLOPHILIN20-3 (CYP20-3) acts as a plastid localised receptor linking OPDA signalling to cellular redox homeostasis in the response to stress in Arabidopsis (Park *et al.*, 2013). We tested whether CYP20-3 also plays a role in seed OPDA signalling under different light conditions. To do this we assessed germination of *cyp20-3* knockout mutant seeds under FR and FR48 treatments, but observed no significant germination increase, even in the presence of norflurazon (Fig. S4). However, we did find that *cyp20-3* seeds were resistant to the germination repressive effect of exogenously applied OPDA under white light conditions (Fig. S4). These results indicate that CYP20-3 is involved in the mechanism by which exogenous OPDA inhibits seed germination, but that CYP20-3 is not required for transducing the OPDA effect under FR light conditions (although we cannot exclude that it may act redundantly with other signalling factors).

Conclusions

The integration of the data presented in this and our previous studies allows us to propose a model in which the germination repression effect of OPDA and ABA under shade light conditions is, at least

partially, modulated by MFT (Fig. 6). We have demonstrated that under FR light conditions that lead to phyB deactivation, accumulation of OPDA or ABA are sufficient to repress germination (*i.e.* the presence of either phytohormone is enough for the complete FR-driven repression of germination). In contrast, under FR48 light conditions, when the effect of phyB deactivation is partially compensated by phyA activation, both OPDA and ABA are required for the complete repression of germination. We show a correlation of this dependence on OPDA and/or ABA to repress germination with the levels of *MFT* expression: When *MFT* is highly expressed (FR light conditions) OPDA and ABA act redundantly whereas when *MFT* is lowly expressed (FR48 light conditions) OPDA and ABA act non-redundantly. Moreover, we also show a correlation of the OPDA and/or ABA requirements of young and old seeds to repress germination under FR light conditions with *MFT* expression: Compared to young seeds, old seeds express *MFT* at a lower level and require both OPDA and ABA to fully repress germination. It is still not obvious why two phytohormone-based repression pathways have evolved to control seed germination. One could argue that, because of the critical importance of germination in the plant life cycle, it has been advantageous to adopt a 'belt and braces' approach to its control. The deployment of two repressor systems may also allow a greater flexibility or fine tuning of the different temporal, spatial and physiological factors that could all be influencing when a seed germinates.

Supplementary data

Figure S1. Relative *AOS*, *OPR3* and *UBQ11* gene expression.

Figure S2. OPDA, JA and JA-Ile accumulation in Col, opr3-1 and opr3-3.

Figure S3. Effect of JA-treatment on germination of *aos aba2-1* and *della* quadruple mutant seeds.

Figure S4. Analysis of *cyp20-3* seed germination.

Table S1. Sequence of primers used in this study

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Figure Legends

Fig. 1. Analyses of gene expression and accumulation of OPDA and ABA. (A) Scheme of the experimental design: after-ripened seeds were imbibed for 4 hours under white-light (WL) and then treated with (i) two consecutive 5 minutes FR and R pulses (FR/R); (ii) only one FR pulse (FR); or (iii) 48 hours of continuous FR irradiation (FR48). Seeds were kept in the dark after light treatments. Samples were collected for analyses at 12 and 24 hours-after-imbibition (hai) as stated in the figure. (B-

C) Relative *AOS* and *OPR3* expression. (D-E) ABA and OPDA levels in FR/R and FR-treated wild-type (Col) and FR-treated mft-2 seeds. Data are means of three and four biological replicates for gene expression and germination assays, respectively, and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

Fig. 2. Germination assays of OPDA and ABA deficient seeds. (A) Wild-type (Col) and *aos* seeds on control and norflurazon (Norf; 50 and 100 μM) supplemented plates under FR/R and FR light. (B) Col, *opr3-1* and *opr3-3* seeds on control and norflurazon (Norf; 50 and 100 μM) supplemented plates under FR/R and FR light. (C) Col, *aos* and *aba2-1* single, and *aos ab2-1* double mutant seeds under FR/R, FR and FR-FR treatments. (D) Germination of *aos aba2-1* seeds treated with OPDA or ABA (1 and 10 μM) under FR light conditions. Germination was assessed 144 hai (seeds had been after-ripened for not longer than 8 weeks). Data are means of four biological replicates and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

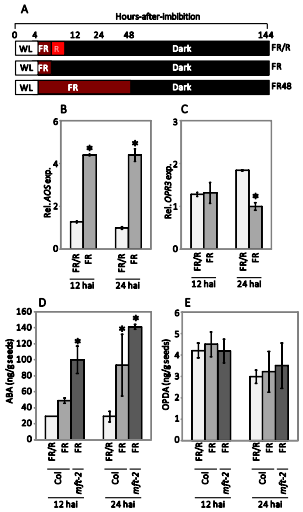
Fig. 3. Effect of GA, OPDA or ABA on germination of mutant seeds under FR light conditions. (A) *aos aba2-1* double mutant seeds on control and Paclobutrazol (PAC; 5 μM) supplemented plates. (B) *rgl1-1 rgl2-2 gai-6 rga-2* quadruple (della4) mutant seeds on control, OPDA and ABA (1 and 5 μM) supplemented plates. (C) *mft-2* mutant seeds on control and OPDA (10 μM) supplemented plates. Germination was assessed 144 hai. Data are means of four biological replicates and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

Fig. 4. *PIF1*, *SOM* and *MFT* gene expression. (A) Relative gene expression in after-ripened wild-type (Col) seeds under FR/R, FR and FR48 light treatments (48 hai). (B) Relative MFT expression in after-ripened Col, *aos*, *aba2-1* and *aos aba2-1* seeds under FR48 light treatment (48 hai). Data presented are the means of three biological replicates and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

Fig. 5. Analyses of long-term after-ripened seeds. (A) Germination (144 hai) of wild-type (Col) seeds upon FR/R and FR treatments on control and norflurazon (Norf; 100 μM) supplemented plates. (B) Germination (144 hai) of Col and *aos* seeds upon FR/R and FR treatments. All seeds were after-ripened for at least nine months before conducting germination assays. (C) Relative *MFT* expression in young and old (8-weeks and 9-months after-ripened, respectively) FR-treated seeds. Data are means of four (for germination) and three (for gene expression) biological replicates and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

Fig. 6. Model of interaction between OPDA, ABA and MFT to repress germination. Treatment with FR light early after seed imbibition deactivates phyB but has no effect on phyA as it has not yet accumulated. Under these conditions endogenous OPDA and ABA fully repress seed germination and promote expression of the germination repressor MFT (A). In the absence of either OPDA and ABA, the action of the remaining phytohormone and the reduced level of MFT is sufficient to fully repress germination (B and C). When both phytohormones are absent, the low level of MFT expression is not sufficient to repress germination (D). This model also explains the partial germination of OPDA or ABA deficient seeds following a FR48 treatment, which activates phyA and deactivates phyB leading to a reduction of *MFT* expression (compare Figs 2 and 4). Similarly, the model explains the partial germination in response to FR treatment of old seeds deficient in ABA or OPDA.

567 Fig. 1



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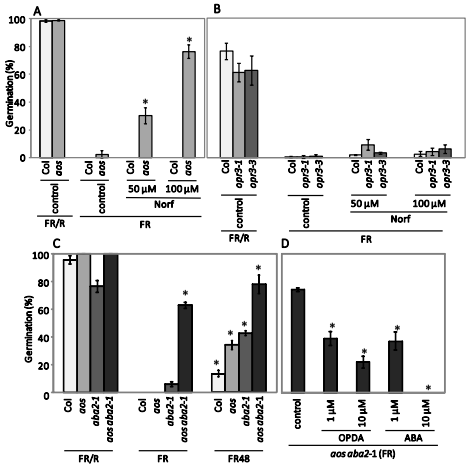
570 Fig. 2



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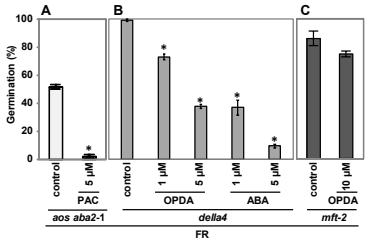
573 Fig. 3



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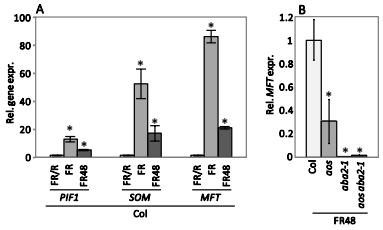
576 Fig. 4


A
Rel. gene expr.
100
80
60
40
20
0
FR/R
FR
FR48
PIF1
SOM
MFT
Col
B
Rel. MFT expr.
1.2
1
0.8
0.6
0.4
0.2
0
Col
aos
aba2-1
aos aba2-1
FR48

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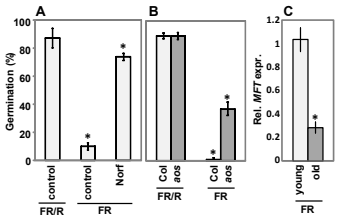
578 Fig. 5


A
B
C
Germination (%)
100
80
60
40
20
0
control
control
Norf
FR/R
FR
Col
aos
Col
aos
FR/R
FR
Rel. MFT expr.
1.2
1
0.8
0.6
0.4
0.2
0
young
old
FR

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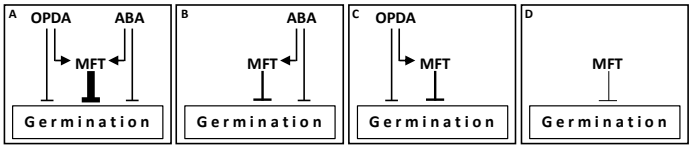
580 Fig. 6



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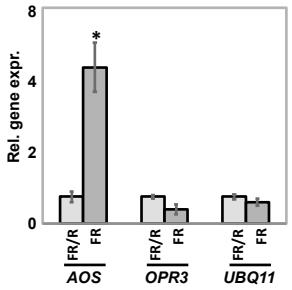

Sup. Fig. 1 Relative *AOS*, *OPR3* and *UBQ11* gene expression. Data extracted from an RNAseq-based transcriptomic analysis performed previously (Vaistij *et al*., 2016) of FR/R- and FR-treated Col seeds (24 hai). Data presented are the means of three biological replicates and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

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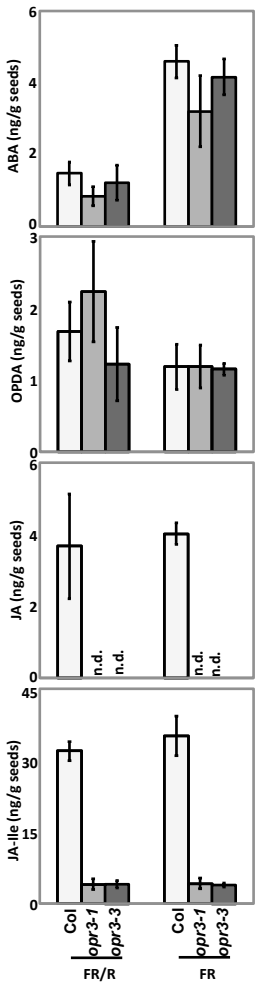

Sup. Fig. 2 OPDA, JA and JA-Ile accumulation in Col, *opr3-1* and *opr3-3* seeds. Seeds were FR/R and FR treated and material collected 24 hai. Data presented are the means of four biological replicates and error bars represent standard deviation. (n.d.: not detected.)

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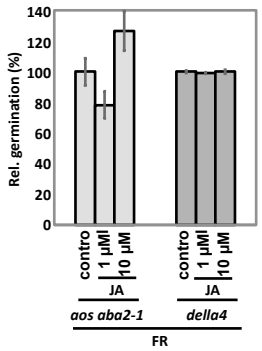
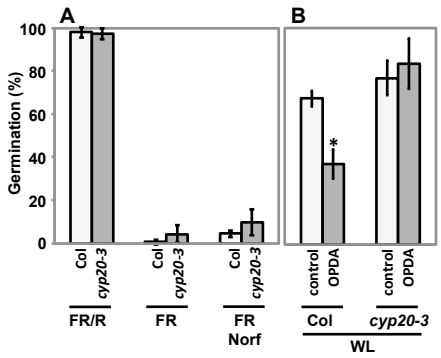


Sup. Fig. 3 Effect of JA-treatment on germination of *aos aba2-1 double* and *della* quadruple mutant seeds. A-B Relative germination (144 hai; FR-treated) of *aos ab2-1* double (A) and *rgl1*-1 *rgl2*-2 *gai*-6 *rga*-2 quadruple (*della4*; B) mutant seeds on control and JA (1 and 10 µM) supplemented plates. Data presented are the means of four biological replicates and error bars represent standard deviation.

586



Sup. Fig. 4 Analysis of *cyp20-3* seed germination. **A** Germination (144 hai) of Col and *cyp20-3* seeds treated with FR/R, FR and FR supplemented with 100 µM norflurazon (Norf). **B** Germination (144 hai) of Col and *cyp20-3* seeds under white light (WL) on control and OPDA (10 µM) supplemented plates. Data presented are the means of four biological replicates and error bars represent standard deviation. Asterisks (*) denote statistical significant difference compared to the respective controls as determined by Student's t-test ($P < 0.05$).

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Table S1 | Sequence of primers used in this study for RT-qPCR in gene expression analyses

Oligo name	Forward	Reverse
AOS	AAGTCAAAGCCGGTGAAAT	CTTACCGGCGCATTGTTTAT
OPR3	TGGACGCAACTGATTCTGAC	CTCATCACTCCCTTGCCTTC
PIF1	TGTCAATGGGATGTGGAATGA	CATCGCCATATGAGGCATGTA
SOM	TCCGGATGTTCGAATTCAAGAT	GCAAAAGGACAATCAGTCCAATC
MFT	ATCACTAACGGCTGCGAGAT	CGGGAATATCCACGACAATC
UBQ11	TTCATTTGGTCTTGCGTCTG	GAAGATGAGACGCTGCTGGT

590